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METHODS FOR THE ISOLATION OF OOCYSTS OF CRYPTOSPORIDIUM FROM SLUDGE AND GIARDIA CYSTS FROM STOOL

The University of Arizona M.S. 1986

METHODS FOR THE ISOLATION OF OOCYSTS OF CRYPTOSPORIDIUM FROM SLUDGE AND GIARDIA CYSTS FROM STOOL

by

Dima Kayed

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THE UNIVERSITY OF ARIZONA

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This thesis has been approved on the date shown below:

Charles P. Gerba — Dec 15, 1986

Charles P. Gerba — Date

Professor of Microbiology/Immunology

DEDICATION

To my family for their constant love, support and understanding.

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ABSTRACT

A method was developed to detect indigenous Cryptosporidium in sewage sludge. The use of sonication, cheesecloth filtration, various flotation ratios of sucrose and large and small volumes of sludge (anaerobically digested) were examined. Optimal recoveries were obtained using 3/5 Sheather's, no cheesecloth filtration and a 1:3 flotation ratio. Cryptosporidium was identified in samples of sludge on the basis of size, shape and reaction with a fluorescently labelled monoclonal antibody. Concentrations ranged from 3.06×10^{2} to 3.87×10^{4} oocysts per gram dry weight.

In an attempt to determine the most effective flotation for the recovery of Giardia cysts from stool six flotation media were evaluated. Methodology variables tested included detergents, speed of centrifugation for concentration of cysts, sonication, flotation ratios and volume of sample. Optimal recoveries for both flotations occurred using sonication, low speed centrifugation, detergents and a 1:10 flotation ratio.

CHAPTER 1

INTRODUCTION

Wastewater Treatment and Sludge Production

Wastewater sludges have been shown to contain a wide variety of microorganisms pathogenic to humans which can be divided into four groups: bacteria, protozoa, helminths, and viruses. Treatment practices are thought to reduce the numbers of these organisms, but evidence indicates that effluents and sludges contain detectable amounts of each of the four groups (Burge and Marsh, 1978). During wastewater treatment a large amount of sludge is generated which may contain significant concentrations of pathogens, metals, toxic chemicals, phosphorus and nitrogen. Microbial pathogens such as protozoa and helminths may be of major concern in sludge because of their environmentally stable cysts or ova.

The objective of wastewater treatment is to reduce the concentrations of specific pollutants and to remove the organic matter, toxic chemicals and human pathogens to ensure the discharge of an effluent which is non-hazardous to both man and the environment. Sewage is most often subjected to the following three treatment processes: 1) primary treatment to remove solids, usually by gravity

settling; 2) secondary treatment, which is designed to remove the demand for oxygen (BOD) and is biological in nature; and, 3) tertiary treatment which utilizes chemical or physical methods to remove inorganic compounds which may include phosphorus and nitrogen as well as pathogenic microorganisms.

Primary sludge is the solid which initially enters the treatment plant and drops to the bottom of a primary clarifier (2-6 hours retention time) or settling tank. This raw sludge, which contains about 60% of the suspended solids, must be stabilized to retard further decomposition and dewatered for ease of disposal. Studies on laboratory and full-scale primary sedimentation tanks have been shown to reduce Entamoeba histolytica cysts in the wastewater by 50% or less. Approximately 50-70% of helminth eggs also settle out (Feachem et al., 1983).

The second and most significant source of sludge comes from secondary treatment. After primary settling, sewage containing dissolved organic compounds is introduced into an aeration tank. Organic compounds in the sewage are decomposed by the microbial population. Since oxygen is necessary for this rapid process aeration chambers are used where air or oxygen can be pumped into the wastewater. The solids including microorganisms are separated from the liquid in a secondary clarifier. The rapid development of microorganisms is stimulated by the

reintroduction of the settled sludge from a previous run. Thus the process derives its name from this inoculation with "activated sludge". The activated sludge system reduces the BOD to between 70-90% and removes approximately 70-97% of suspended solids. The activated sludge process has little effect on protozoal cysts and helminth eggs but substantial numbers of eggs are removed in the secondary settling tanks. Removals of 80-100 percent of helminth eggs have been reported in complete activated sludge treatment plants (Feachem et al., 1983).

Sludge Treatment

The main problem associated with sludge is a means for its final disposal. Sludge disposal is not only an economical problem but also an environmental problem because it is aesthetically displeasing and contains potentially harmful chemicals and microorganisms. Methods which address these issues and are included in sludge treatment processes include anaerobic or aerobic digestion, liming and removal of water by thickening or dewatering (Peirce and Vesilind, 1983).

Aerobic stabilization is similar to the activated sludge system. It involves large energy use for aeration and mixing of the waste activated sludge. In temperate climates, retention times of 15-30 days, and in cold climates up to 50 days are necessary to provide adequate stabilization of the sludge. This process occurs in a

variety of large devices (oxidation ditches, trickling filters, etc.) (Bruce et al., 1983).

Anaerobic digestion is used to further process the sewage sludge produced by primary and secondary treatments. Anaerobic digesters contain high amounts of suspended organic matter. Favorable levels are between 20 and 100 g/l, (Atlas, 1984). Much of the suspended material is bacterial biomass and viable counts can be as high as 10^{9}-10^{10} bacteria per ml. Fungi and protozoa, mainly ciliates don't seem to play a significant role in anaerobic digestion since they are present in very low numbers (Atlas, 1984). Anaerobic digestion is a two step process. In the anaerobic digesters, a hearty group of anaerobic bacteria (acid formers) convert the organic materials and microbial biomass to fatty acids, carbon dioxide, and hydrogen gas. The carbon dioxide and hydrogen are further degraded by a group of strict anaerobes called methane formers. Thus the final products obtained in an anaerobic digester are a gas mixture (70% methane and 30% carbon dioxide), microbial biomass and a non-degradable residue (Atlas, 1984). Optimal operation of anaerobic digesters require control of several variables which include retention time, temperature (35-37 ^{o}C), pH (6.0-8.0, 7.0 optimal) and carbon to nitrogen (C:N) and carbon to phosphorus (C:P) ratios (Atlas, 1984). An anaerobic digester which is operating optimally will

yield a reduced volume of sludge compared to the starting material; however, the product still causes odor and water pollution problems and, therefore, precautions should be taken for its disposal. Aerobic composting may alleviate this problem and render it suitable for disposal in landfills.

Sludge digestion may reduce pathogenic microorganisms depending on the type of digestion and organisms of interest. Batch digestion at 32 ^{o}C for 28 days may leave many helminth eggs and some excreted viruses and bacteria, whereas 120 days of unheated digestion in warm climates will only leave helminth eggs and a few remaining bacteria and viruses (Feachem et al., 1983). The only digestion process (50 ^{o}C for 13 days) which leaves a thoroughly pathogen-free sludge is termed batch thermophilic digestion. Most mesophilic (32 ^{o}C for 28 days) and thermophilic digestion as well as composting may eliminate Giardia cysts. In Chicago (U.S.A.) Giardia cysts were not found in anaerobically digested sludges despite their presence in raw sewage (Fox and Fitzgerald, 1979).

Other processes, such as lime stabilization and dewatering, may also remove pathogens. Lime stabilization is achieved by adding lime (hydrated lime $Ca(OH)_2$ or quicklime (CaO) to sludge and raising the pH to 11 or above. This helps to reduce odor and aids in the

destruction of pathogens. The pH, however, does drop over time (Peirce and Vesilind, 1983). Sludge thickening is a process whereby the solid concentration is increased and total sludge volume is decreased thus facilitating handling. Drying sludge in open beds for 2-3 months will remove the great majority, and possibly all, of the excreted viruses and bacteria, at warm temperatures above 20 ^{o}C, Protozoal cysts will also be destroyed (Feachem et al., 1983). Only persistent helminth eggs, such as Ascaris, Trichuris and Taenia have been shown to survive under these conditions (Feachem et al., 1983). Other unheated dewatering processes such as vacuum filtration, centrifugation, and pressure filtration will have little effect on pathogen survival.

Sludge cannot be easily disinfected by chlorination, due to its solid nature and high organic content. Methods more commonly used include pasteurization, composting, heat-drying, and liming. Heat drying seems to be effective but costly. Liming is used mainly for stabilization of raw sludge for disposal at landfills. Composting is apparently effective in pathogen destruction provided that temperatures are high (> 60 ^{o}C) and the composting system is well aerated (Burge and Marsh, 1978; Feachem et al., 1983). Composting involves the mixing of sludge with a carbon source (such as refuse or saw dust) to achieve a C:N ratio of about 20-30 and a

moisture content of approximately 20-60 percent. Pasteurization is effective in killing pathogens but does not stabilize the sludge (Burge and Marsh, 1978).

Sludge Disposal

The choices for the ultimate disposal of sludge are limited to air, water and land. Controls on air pollution complicate incineration. Disposal of sludges into deep waters such as oceans is decreasing due to adverse detrimental effects on the aquatic ecosystem. Land disposal involves either dumping the sludge into landfills or spreading it over the land and allowing for natural biodegradation to assimilate the sludge back into the environment. Only incineration and land disposal are commonly used due to environmental and cost considerations (Peirce and Vesilind, 1983). Incineration involves the conversion of organics in sludge to H_2O and CO_2 and the inorganics drop out as residue ash. Land spreading, the second means of disposal, depends on the ability of the land to absorb and assimilate the sludge. This, in turn, is dependent on such variables as soil type, vegetation, rainfall, slope, etc. The characteristics of the sludge also will influence the capacity of the soil to assimilate it. Basically, sandy soils with lush vegetation, low rainfall and gentle slopes have proven most successful (Peirce and Vesilind, 1983). Most unsuccessful land

application is traced back to overloading the soil (application rate 100 dry tons/acre/year).

During land disposal of sludge, data has shown that enteropathogens may survive from days to years in water, soil and on plants. Entamoeba histolytica cysts can survive in soils from 6-8 days, on plant surfaces less than 3 days, and in the water from 8-40 days (Burge and Marsh, 1978). Ova of the intestinal worm, Ascaris lumbricoides, have been shown to survive in garden soils for up to 7 years and on plant surfaces (vegetables and fruits) in the field less than 35 days (Burge and Marsh, 1978). Survival periods for Giardia cysts are presently unknown. Best estimates of cyst survival in the environment indicate that Giardia may be similar to Entamoeba histolytica.

Most sewage related disease outbreaks have been associated with the use of raw sewage, raw sludges, or "night soils" (human feces) on food crops consumed raw, or the contamination of drinking water from septic tanks (Burge and Marsh, 1978). If sludge is not rendered suitable for final disposal and microorganisms are indeed present, this could be a source for spread of disease. Currently, there is no regulation governing the application of solid wastes to crops and crop lands in the United States and Canada. Although some states have implemented regulations for wastewater reuse, the reuse

on disposal of sludges is less well defined. Some states allow the use of completely treated disinfected sewage effluent on fruits and vegetable crops to be eaten raw, whereas other states ban the use of any sewage effluents on crops. In the future, state and/or federal regulations on land application of sludges may be necessary to provide additional protection of the public health.

Waterborne Outbreaks by Protozoans

Waterborne disease is a growing problem in the United States. Between 1946 and 1980, 672 waterborne outbreaks affecting more than 150,000 persons were reported in the United States (Lippy and Waltrip, 1984). In about 50% of these outbreaks, the etiologic agent was not identified. Known causative agents include bacteria, viruses, chemical agents and parasitic protozoa (Lippy and Waltrip, 1984). Among the protozoa, Giardia, Entamoeba and a newly recognized microorganism Cryptosporidium have been identified in waterborne outbreaks. Detailed discussion will be limited to Giardia, since most of the information available in recent years has focused on this organism.

Giardiasis is endemic in many countries of the world including the U.S. Giardia cysts find their way into water supplies from human sewage and from animals. Animals may be able to pick up the disease from human waste disposal practices and subsequently contaminate a

water supply. Accidental mixing of sewage and water has occurred and sewage and sewage sludges have been known to contain G. lamblia cysts (Craft, 1982). Complete sewage treatment, which includes anaerobic digestion to eliminate the cysts, may protect surface water sources from contamination.

Waterborne transmission of Giardia was implicated as early as 1946 during an investigation of an outbreak of amebiasis attributed to sewage contamination of a water supply in a Tokyo apartment building. Entamoeba histolytica and Giardia lamblia were recovered from 96 (64%) and 116 (77%) occupants, respectively (Davis and Ritchie, 1948). During the years 1965-1981, 53 waterborne outbreaks of giardiasis affecting more than 20,039 persons occurred in the United States. During the period 1972-1981 G. lamblia was the most commonly identified pathogen in waterborne outbreaks with 15% of all such outbreaks and 26% of all disease attributed to this organism (Craun, 1984).

Sewage contamination has greatly contributed to the transmission of Giardia through waters. The inadequate treatment of drinking waters has likewise been important. An outbreak of waterborne giardiasis occurred in Aspen, Colorado (1965-66) infecting 11% of 1064 skiers (Lin, 1985). The city's water came from a mountain creek and three wells. The latter was thought to have been

contaminated by leaking sewage from which G. lamblia cysts were isolated. A large outbreak of waterborne giardiasis, where for the first time G. lamblia cysts were actually recovered from water occurred in Rome, New York (1974-75) affecting between 4800-5300 people. The city of Rome used a surface water source and chlorination was the only treatment. Outbreaks also occurred in Camas, Washington (1976), and Berlin, New Hampshire (1977). Both utilized filtration and chlorination of water supplies (Craun, 1984; Lin, 1985). It appears that most outbreaks and disease occur due to consumption of untreated surface water with disinfection as the only treatment. Ineffective filtration also has been responsible for waterborne outbreaks and disease (Craun, 1984).

The following factors enhance the likelihood of Giardia transmission by water and hence the likelihood of disease: 1) animal reservoirs such as beavers and muskrats; 2) long survival times of cysts in water (1-3 months); 3) high concentrations of cysts in feces; and, 4) their low infective dose (Sobsey and Olson, 1983).

Inactivation of Giardia in Water

Disinfection studies indicate that chlorine disinfection with normal dosage (10-30 milligrams per liter) and contact time (at least 1 hour) is insufficient to destroy G. lamblia cysts. A higher dosage of chlorine

with longer contact time is required in water treatment systems that do not utilize sedimentation and filtration to remove the cysts (Lin, 1985). Chemical disinfection may be difficult for sludge due to its solid nature and organic content. Methods more commonly used include composting, heat drying, pasteurization, and liming as discussed previously.

Giardia lamblia

The Organism

Giardia lamblia is a flagellated protozoan belonging to the class Zoomastigophorasida, order Diplomonadorida and family Hexamitidae (Levine, 1979). It has both a trophic and cystic stage. The length of the trophozoite ranges from 9 to 21 um, the width from 5 to 15 um and the thickness from 2 to 4 um. These trophozoites usually inhabit the duodenum and jejunum of humans and appear in the feces of diarrheic individuals. In non-diarrheic individuals, the parasite encysts and cysts are shed in the stool. Cysts are characteristically oval to ellipsoid and slightly asymmetric in shape, ranging from 8-14 um in length and 7-10 um in width. Transmission of giardiasis from host to host may occur by ingestion of Giardia cysts in contaminated drinking water and foods or by direct contact with infected persons (Lin, 1985).

Pathogenesis and Treatment

Symptoms may range from mild diarrhea, malaise, weakness, fatigue, dehydration, weight loss, distention, flatulence, anorexia, cramp-like abdominal pain and epigastric tenderness to steatorrhea and malabsorption. This is due to the attachment of G. lamblia to the surface of epithelial cells of the intestine by means of its ventral sucking disc. This dense coating of trophozoites on the intestinal epithelium interferes with the absorption of fats and other nutrients, thus contributing to the clinical syndrome, giardiasis. The parasite does not lyse host cells but appears to feed on mucus secretions which may cause a disturbance of intestinal function or low grade inflammation of the duodenal or jejunal mucosa. Due to these abnormalities the patient suffers acute or chronic diarrhea. Stools may be watery, semi-solid, greasy, bulky, and foul smelling without blood. Drugs available in the United States for the treatment of giardiasis are Atabrine, Flagyl, and Furoxone (Lin, 1985).

Cryptosporidium

Cryptosporidium is a coccidian protozoan parasite. The oocyst is spherical and ranges in size from 4-6 um. It is a genus in the family Cryptosporidiidae, suborder Eimeriina, order Eucoccidiidae, subclass Coccidia, class Sporozoa, phylum Apicomplexa (Levine et al., 1980). The

suborder Eimeriina contains over 1500 species with the majority of these belonging to the genera Eimeria and Isospora, both of which are intracellular parasites primarily infecting the intestinal tract of vertebrates (Tzipori, 1983).

Initially, Cryptosporidium was thought to be host specific and new species were named as they were found in new hosts. Research, however, has shown that the mammalian species could easily be transmitted between a variety of hosts. Tzipori et al. (1980) found that Cryptosporidium isolated from calves with diarrhea infected seven different species of mammals. It also has been suggested that Cryptosporidium may be a single-species genus (Tzipori et al., 1980).

The lifecycle of Cryptosporidium consists of six major developmental phases: excystation (release of infective sporozoites), merogony (asexual multiplication), gametogony (gamete formation), fertilization, oocyst wall formation, and sporogony (Current, 1984; 1985). It is monoxenous, whereby development occurs in a single host (Navin and Juranek, 1984). The lifecycle of this organism begins when sporulated oocysts in the feces are ingested by a potential host. Excystation of the cysts to release sporozoites occurs in the intestine where type I meronts with six to eight merozoites are formed. These either recycle or become type II meronts containing four

merozoites. These type II meronts develop into either micro, or male gametocytes containing sixteen microgametes or macrogametes. The microgamete fertilizes the macrogamete to form a zygote. Approximately 80% of the zygotes form thick-walled oocysts which can pass down the gut and transmit the infection to a potential host via the fecal-oral route. The remaining 20% do not form the oocyst wall and their sporozoites are surrounded by a single unit membrane. The sporozoites of these oocysts are autoinfective and can reinitiate the cycle. The presence of type I merozoites which can reinitiate the cycle and the presence of autoinfective oocysts may explain why such a small inoculum can produce an overwhelming infection in a susceptible host in the absence of repeated exposure to the oocysts (Current, 1984; 1985). The Cryptosporidium oocyst releases sporozoites in response to bile salts within the gastrointestinal tract of a new host (Fayer and Leek, 1984).

The organism was first described from the gastric mucosal glands of the laboratory mouse by Tyzzer (1907). Clinical illness was first associated with this organism in 1955 when Slavin (1955) reported severe diarrhea among turkeys infected with Cryptosporidium. Until recently, Cryptosporidium, was considered to be an uncommon infection in animals and in man it was thought to be a

little known opportunistic pathogen outside its normal host range (Current, 1984; 1985; Tzipori, 1983). Cryptosporidium is now an important cause of gastroenteritis and diarrhea in many animal species, and especially in calves, lambs, and man (Current, 1984; 1985; Tzipori, 1983). In immunocompetent persons it produces a short-term flu-like illness, however, in immunodeficient persons, and especially those with acquired immune-deficiency syndrome (AIDS), a severe prolonged diarrhea occurs which is life threatening (Current, 1984; 1985; Current and Reese, 1983). Cryptosporidium is also considered a zoonotic protozoan which can be transmitted directly from animals to man (Current, 1985).

Clinical Manifestations

Cryptosporidium causes a mild to severe diarrhea with anorexia and weight loss in animals 1 to 3 weeks of age, with high morbidity and low mortality, due to dehydration and malabsorption (Current, 1984; Navin-Juranek, 1984). In poultry, Cryptosporidium has been associated with intestinal and respiratory disease. Outbreaks of respiratory disease have also been reported in broiler chickens and turkeys with high morbidity and mortality (Current, 1985). Clinical manifestations have been found to be dependent on the following variables: the species involved, its age, and its immune status.

Young animals, especially neonates, are more prone to infection than older animals. It has been suggested that adult animals may develop immunity to the organism but cannot provide passive immunity to their offspring (Navin and Juranek, 1984).

The first case of human cryptosporidiosis was reported in 1976 (Nime et al., 1976). Between 1980 and 1982, 11 similar cases were reported (Angus, 1983). All of these cases were observed in immunocompromised individuals. In humans, as in animals, the major determinant of disease severity is the immunologic status of the host. Asymptomatic and self-limited infection occur in patients with normal immune function whereas immunocompromised patients develop severe life-threatening diarrhea (Casemore et al., 1985; Navin and Juranek, 1984). Between 1980 and 1984, 44 cases of prolonged infection in immunocompromised individuals were reported to the Centers for Disease Control (CDC) (Current, 1984). Wolfson et al. (1985) found that Cryptosporidium was a common non-viral cause of diarrhea in immunocompetent persons, especially children during the summer and fall. Cryptosporidium is world-wide (Levine, 1984).

Symptoms of the disease in humans are characterized by profuse, watery diarrhea without gross or microscopic blood. The diarrhea in immunosuppressed patients is accompanied by enormous fluid losses. Other

symptoms include mild epigastric cramping pain, nausea, vomiting, anorexia, fever, weight loss and malaise. Fever among immunosuppressed patients is difficult to interpret due to other concurrent infections (Navin and Juranek, 1984; Wolfson et al, 1985). Cryptosporidium has been found in the pharynx, stomach, esophagus, duodenum, jejunum, ileum, colon, appendix and rectum of immunocompromised patients (Current, 1984).

Few studies on the prevalence of Cryptosporidium have been done. The prevalence of this organism in the United States is not known. An Australian survey found Cryptosporidium in the stools of 36 (4.1%) of 884 patients hospitalized for gastroenteritis and 320 other patients without gastroenteritis were not infected (Tzipori et al., 1983). The latter finding suggests that asymptomatic carriers may not be common in the general population (Navin and Juranek, 1984). Recent studies have shown a high prevalence for infection during the summer months, (Tzipori et al., 1983; Wolfson et al., 1985), the reason being unknown. Overall reports show the prevalence of Cryptosporidium isolations from stool specimens to be about 2% (Casemore et al., 1985; and Jokipii et al., 1985).

Epidemiology

The epidemiology of Cryptosporidium is poorly defined because this organism has only recently been

described in humans. Studies of experimental infections in animals have shown that Cryptosporidium is transmitted by oocysts that are infective immediately after passage in the feces.

The principal mode of transmission of Cryptosporidium is the fecal-oral route and may occur by either direct or indirect contact with contaminated feces. Direct transmission can occur during sexual practices involving oral-anal contact. Indirect contact usually occurs by exposure to fecal contaminated environmental surfaces, food, water, or fomites (Current, 1984; Navin and Juranek, 1984). Outbreaks of Cryptosporidium have occurred in day-care centers (Alpert et al., 1986; Centers for Disease Control, 1984a; Wolfson et al., 1985), hospitals (Current, 1985), and through contact with infected animals. Cryptosporidium has recently been implicated as a possible cause of traveller's diarrhea (Ma et al., 1985; Sterling et al., 1986). Studies have demonstrated that Cryptosporidium can initiate disease across species barriers, thus animals are a potential source of human infection. The organism has been found in association with other enteropathogens such as enterotoxigenic Escherichia coli and rotavirus (Mata et al., 1984; Mathan et al., 1985; Moon et al., 1978; Tzipori et al., 1981). Cryptosporidium has been found in association with Giardia lamblia, a waterborne parasite

(Jokipii et al., 1985; Wolfson et al., 1985). Cryptosporidium was identified as the etiologic agent in an outbreak of gastroenteritis in a Texas community in July, 1984. The outbreak was traced to the contamination of an artesian well by sewage (D'Antonio et al., 1985).

Infectivity of Cryptosporidium Oocysts

Cryptosporidium oocysts are extremely hardy and may survive for long periods within the environment (Navin and Juranek, 1984). They retain their infectivity for up to 6 weeks at room temperature if kept moist (Current, 1984). The oocysts are extremely resistant to a variety of disinfectants that have been used in hospitals and laboratories (Tzipori, 1983). Infectivity of oocysts is completely destroyed by ammonia, formal saline (Campbell et al., 1982), freeze-drying, and exposure to temperatures below freezing (0 ^{o}C) and above 65 ^{o}C for 30 minutes (Tzipori, 1983).

Detection Methods for Giardia and Cryptosporidium In Clinical Specimens

Two methods widely used for detecting G. lamblia cysts in patients are mucosal impression smears and stool examinations. The direct saline wet-mount method of specimen examination is the most popular. Cultivation is least used (Lin, 1985). There is no standard procedure recommended for fixing, preserving and staining stool

samples. Stool samples can be concentrated by zinc-sulfate flotation or formalin-ethylacetate sedimentation, the former being the preferred method for cyst recovery. Polyvinyl-alcohol, Schaudinn's and formalin fixation are used to preserve Giardia cysts (Lin, 1985). Samples may also be preserved in Lugol's iodine. Methods such as membrane filtration, excystation, electron microscopy and the use of fluorescent-antibody have improved the detection of G. lamblia (Lin, 1985).

Giardia In Water

Detection of Giardia cysts in water is more difficult than detection in clinical specimens because of low numbers in the former. A method which includes concentration, separation and microscopic examination of cysts in drinking water has been described by Jakubowski (1984). Sauch (1985) developed a method for the detection and identification of Giardia cysts using indirect immunofluorescence. The antibodies in this procedure were applied on membrane filters and fluorescent microscopy was used.

Diagnosis and Detection of Cryptosporidium

Diagnosis of Cryptosporidium in humans was initially made by microscopic examination of intestinal biopsies with the use of light or electron microscopy (Navin and Juranek, 1984). Recent advances which have

made it possible to detect the organism in stool specimens include Sheather's sugar flotation, modified acid-fast and negative staining (Current, 1985). The most widely used technique to concentrate Cryptosporidium is Sheather's sugar flotation which involves the use of a dense sugar solution having a greater specific gravity than that of the oocysts allowing these oocysts to rise to the surface of the solution. The traditional method of performing this flotation involves placing a coverslip on top of a centrifuge tube with a mixture of stool and sugar solution and after centrifugation placing the coverslip along with the oocysts that have adhered to it onto a glass slide for microscopic examination. A modified method involves capping the centrifuge tube with the mixture, centrifuging and then transferring a sample from the supernatant layer onto a microscope slide for examination. Iodine stained wet mounts can also be used (Navin and Juranek, 1984). Various acid-fast stains, however, have been found to be more useful especially in distinguishing the similarly sized yeast which stain blue-green from Cryptosporidium which stain red (Navin and Juranek, 1984). Garcia et al. (1983) reported best results using Sheather's sugar flotation concentration, Giemsa, Kinyoun acid-fast, Ziehl Neelson acid fast and modified acid fast stains. Garcia (1983) also reported that the most effective technique was use of a modified acid-fast stain on concentrated 10%

formalin stool material after treatment with KOH. In another study, Ritchie-formalin ethylacetate sedimentation (F/EA) plus a modified cold Kinyoun acid-fast stain (MCK) was found to be as sensitive as the Sheather's sucrose flotation (SSF) technique for the detection of Cryptosporidium oocysts (McNabb et al., 1985). Although many stains have been tried, diagnosis is still difficult due to the small size of the oocyst. Recently, however, a monoclonal antibody conjugated to fluorescein has been very successfully used in the rapid detection of Cryptosporidium (Sterling and Arrowood, 1986). In another study, a combination of ether phosphate buffered saline sedimentation and percoll-discontinuous density gradient centrifugation was found to provide a simple and efficient method of concentrating and cleaning oocysts from fecal-debris and intestinal contents of animals (Waldman et al., 1986). Stool specimens suspected of containing oocysts can be preserved in 5% or 10% formalin or placed in 2.5% potassium dichromate which after some time allows sporozoites within the oocysts to be more easily recognized since the oocyst residuum disappears during this period. Oocysts preserved in potassium dichromate remain viable (Navin and Juranek, 1984).

Cryptosporidium in Water

Little work has been done to elucidate the epidemiology of Cryptosporidium in the environment and to

determine its prevalence. A filtration system based upon EPA techniques for isolating Giardia, has recently been improved upon for isolating Cryptosporidium from the aquatic environment (Musial, 1985). Sheather's flotation employing detergents was used for sample clarification. Detection of these oocysts was made possible due to use of direct immunofluorescence employing a monoclonal antibody on membrane filters (Rose et al., 1986).

Cryptosporidium concentrations were found in ranges from 60 to 52,000 oocysts/gal in treated and raw sewage (Rose et al., 1986). This will hopefully provide a method to further investigate the occurrence of Cryptosporidium in the environment.

Parasites in Sludge

Parasites have been isolated from sludges utilizing a variety of methods. Methods used to isolate and concentrate ova and cysts in sludges include flotation procedures such as zinc sulfate and sucrose where the cysts float in the high density solutions, and sedimentation. In some cases, straining the sample through a sieve or filtering through gauze to reduce the amount of organic debris as well as the use of detergents (7X) is recommended (Arther and Fitzgerald, 1981). After layering a volume of sample on a flotation medium and

centrifuging, the supernatant layers are collected and washed with a detergent via centrifugation. Final pellets are resuspended in a detergent and ready to be examined under the microscope. More work, however, needs to be done to evaluate the efficiencies of current methodologies and develop new and better procedures.

Giardia and Cryptosporidium in the Environment

Cryptosporidium and Giardia share similarities which include environmentally resistant cyst stages, fecal-oral transmission and animal reservoirs. Both may be waterborne, causing outbreaks of waterborne disease if water supplies are contaminated. Oocysts of Cryptosporidium have been detected in secondary sewage effluent and in samples from rapid sand filters of a drinking water treatment plant (Rose et al., 1986). Giardia has also been isolated from sewage and sewage sludges as mentioned previously (Craft, 1982). Little work has been done to determine the prevalence of these parasites in the environment. An improved filtration system for Cryptosporidium has been used successfully to detect oocysts in water samples (Rose et al., 1986). This same system may be useful for other parasites particularly Giardia. Appropriate flotation and detection methods, however, need to be developed.

This research was undertaken to develop a method to detect parasites in sludges using Cryptosporidium as a

model. Oocyst detection was facilitated by the employment of the direct immunofluorescence technique with a monoclonal antibody (Sterling and Arrowood, 1986).

Objectives

1. Development of a method for the detection of Cryptosporidium in sludge.
2. Examination of various sludges for natural isolates of Cryptosporidium.
3. Evaluation of various flotation media for the recovery of Giardia.

CHAPTER 2

MATERIALS AND METHODS

Source of Giardia Cysts and Antibody

Giardia cysts of human origin were obtained from stool specimens stored in 10% formalin at 4 °C. The Giardia monoclonal antibody was produced to the G. lamblia cyst and was recovered from cell culture supernatant fluid. The antibody was directly conjugated to FITC and a 1:5 antibody working solution in PBS and 0.1% sodium azide was used. This monoclonal antibody was obtained from Dr. John Riggs (California State Health Laboratory, Berkley, CA).

Use of Membrane Filtration for Giardia Cyst Enumeration

A clean stool sample seeded with Giardia was used to determine a reliable method for the recovery and detection of cysts on membrane filters. Triplicate counts were made on (13 mm) 3.0 um and 5.0 um pore size, polycarbonate membrane filters (Nuclepore, Pleasanton, CA). Distribution of the cysts was determined on the 3.0 um filter. Filtrate counts from both filters were obtained by passing the filtrates through a 1.2 um pore size filter. The Giardia monoclonal antibody was used to detect the cysts.

Effect of Flotation Media on Giardia Cyst Recovery

General Procedure

Approximately 2-3 ml of the stool specimen was washed twice with deionized water (DI water) and concentrated by centrifugation (1400 x g for 10 min) in a 50 ml conical tube to obtain a single pellet. The pellet was resuspended in a volume of detergent solution [DI H_2O with 1% tween 80 and 1% sodium dodecyl sulfate (SDS)]. Samples were homogenized at a low speed for 2 minutes (Vitris "45" Gardiner, New York). One drop of antifoam A (Sigma Chemical Co., St. Louis, MO) was added to facilitate recovery of each sample. This was washed by centrifugation at 1400 x g for 10 minutes and then resuspended back into the detergent solution (1% tween 80 and 1% SDS) or deionzed water.

The sample was vortexed and 1 ml of this sample was layered onto 9 ml of six different flotation media to obtain a 1:10 ratio. The following media were tested: zinc sulfate (specific gravity 1.18-1.20) (Davidsohn and Henry, 1974; Fox and Fitzgerald, 1981), potassium citrate (40%, specific gravity, 1.16), percoll-sucrose (9 ml percoll, 1 ml 2.5 M sucrose: 10 ml 0.25 M sucrose, specific gravity 1.09), full strength Sheather's solution (500 gm sucrose, 320 ml distilled water and 9.7 ml liquid phenol, specific gravity 1.29), 4/5 Sheather's solution

(specific gravity, 1.24) and 3/5 Sheather's solution (specific gravity, 1.17).

In most cases controls (3) were taken from the homogenized preparation. One ml of each was placed in a 15 ml conical tube before layering onto the six flotation media, after layering on three flotation media and at the end of the procedure. Each of the controls were resuspended in 1% PBS-tween 80 up to a volume of 10 ml. The flotation tubes were centrifuged at 1400 x g for 10 minutes and the supernatants were recovered. All recovered supernatants were washed separately two times by dilution in 40 ml of 1% tween 80 and 1% phosphate buffered saline (PBS). Supernatants of potassium citrate were washed in PBS or deionized water and those of zinc sulfate were washed in 1% tween 80 or deionized water to eliminate precipitation at the interface. After the final wash, the excess aqueous solution was aspirated off and the pellets were placed in 15 or 50 ml conical tubes. The pellets were resuspended in 1% tween 80-PBS up to a volume of 10 ml. Turbidities were determined for each of the samples on a turbidimeter (Model 2100 A, Hach Company, Loveland, CO).

Portions of each sample, (1 ml) were filtered either directly or after 1:10 dilutions through 13 mm cellulose nitrate filters or polycarbonate filters with a pore size of 5 um. The filters were stained with 0.1 ml

of a working solution of monoclonal antibody, incubated in a 37 °C incubator for 30 minutes, washed with PBS-tween or distilled water and cysts were enumerated on the filter using fluorescent microscopy. In some cases, after washing with PBS-tween or distilled water, a counterstain Evan's blue (0.003%) was added to the filter for 10 minutes. The sample was then drained of Evan's blue, dehydrated by treatment with an aqueous ethanol series and mounted on a slide for microscopic examination (Sauch, 1985). This counterstain facilitated the counting procedure by quenching much of the background fluorescence due to non-specific binding of the antibody. In addition, intracellular cyst structures were stained.

Percent recoveries for the six different flotation media were determined by total number of cysts recovered after flotation divided by the average number of total cysts obtained from the three controls.

In order to determine whether detergents affected cyst recovery, the basic method was followed as outlined in the general procedure. Detergents and antifoam A, however, were not used. In this set of experiments two flotations 4/5 Sheather's and 40% potassium citrate were evaluated. Two controls were also taken, one before layering the sample onto flotation and one following the layering procedure. Flotations were centrifuged at 1400 x g for 10 minutes and final pellets were resuspended in 1%

PBS-tween 80 up to a volume of 10 ml. Filters were stained and enumerated accordingly.

Effect of Sonication and Low Speed Centrifugation on Cyst Recovery

Sonication and low speed centrifugation were used to increase cyst recovery. The basic method was followed as outlined in the general procedure (pg. 28) and two flotations, 4/5 Sheather's and 40% potassium citrate were evaluated. After homogenization and addition of antifoam A the sample was split up into two tubes and centrifuged at 1400 x g for 10 minutes to eliminate the antifoam. The pellets were recovered and one pellet was resuspended in distilled water and the second in 1% SDS-tween. Both tubes were placed in a water bath sonicator (25 KHz) (Ultrasonic Cleaning System E-module Branson Cleaning Equipment Co., Shelton, CT) for four minutes. One ml of the sample was taken from the tube in distilled water and layered onto 9 ml of 40% potassium citrate to give a 1:10 ratio. A control was also taken from the same tube. One ml of the sample in 1% SDS-tween was layered onto 9 ml of 4/5 Sheather's and a control was also taken. Both flotation tubes were centrifuged at 600 x g for 2 minutes and supernatant portions were recovered. The supernatant portion from potassium citrate was washed in distilled water rather than PBS-tween. Final pellets were resuspended in 1% PBS-tween and examined in the same

manner as discussed previously. Evans blue (0.003%) was used as a counterstain to reduce background fluorescence. Turbidities were recorded and percent recoveries determined in the same manner as described in the general procedure.

Effect of Sample to Flotation Ratios on Cyst Recoveries

In this set of experiments, various ratios of sample to flotation media were evaluated for cyst recoveries. The procedure included homogenization, sonication, detergents and low speed centrifugation (600 x g for 2 min). A 5 ml volume of sample was layered onto 10, 15 and 25 ml of flotation media to give a 1:2, 1:3 and a 1:5 ratio, respectively. Two controls were taken, one before the layering procedure and one following it. Potassium citrate and 4/5 Sheather's flotations were evaluated for percent recovery of cysts as previously described. Turbidities were also recorded.

Detection of Cryptosporidium in Sludge Preparation of Oocysts and Antibody

Cryptosporidium oocysts were supplied by Dr. Charles R. Sterling, Department of Veterinary Science, University of Arizona and his laboratory. The following briefly describes the procedure used. Two to four day old calves were orally infected with 10^8 Cryptosporidium oocysts. Feces were collected at the onset of oocyst shedding and processed by sieving through stainless steel

mesh screens. The sieved feces were pelleted and layered onto discontinuous Sheather's gradients. The mixture was centrifuged at 1700 x g for 15 minutes followed by recovery of oocysts floating within the top gradient layer. The oocysts were washed in phosphate buffered saline (PBS) and further purified on an isopycnic percoll gradient (Arrowood and Sterling, 1987). Cleaned oocysts were resuspended in 2.5% potassium dichromate followed by resuspension in 10% formalin for seeded studies.

The antibody used in this study was supplied and developed by Dr. Charles R. Sterling and his laboratory. The IgG1 monoclonal antibodies (MAB) to the oocyst wall of Cryptosporidium were derived by immunizing Balb/c mice with intact oocysts and isolated oocyst walls. The cloned IgG1 immunoglobulin from mouse ascites was purifed by ion-exchange chromatography and directly labeled with fluorescein isothiocyanate (MAB-FITC). This stock antibody was filtered and gave an indirect immunofluorescent titer of 1:512. To make the antibody working solution, 0.2 ml of stock antibody was diluted 1:10 in 9.8 ml PBS with 0.1% sodium azide.

Method for the Detection of Oocysts in Sludge

The method used for seeded studies and enumeration of indigenous Cryptosporidium oocysts in sludges is as follows (Fig. 1). Twenty ml of tap water was seeded with

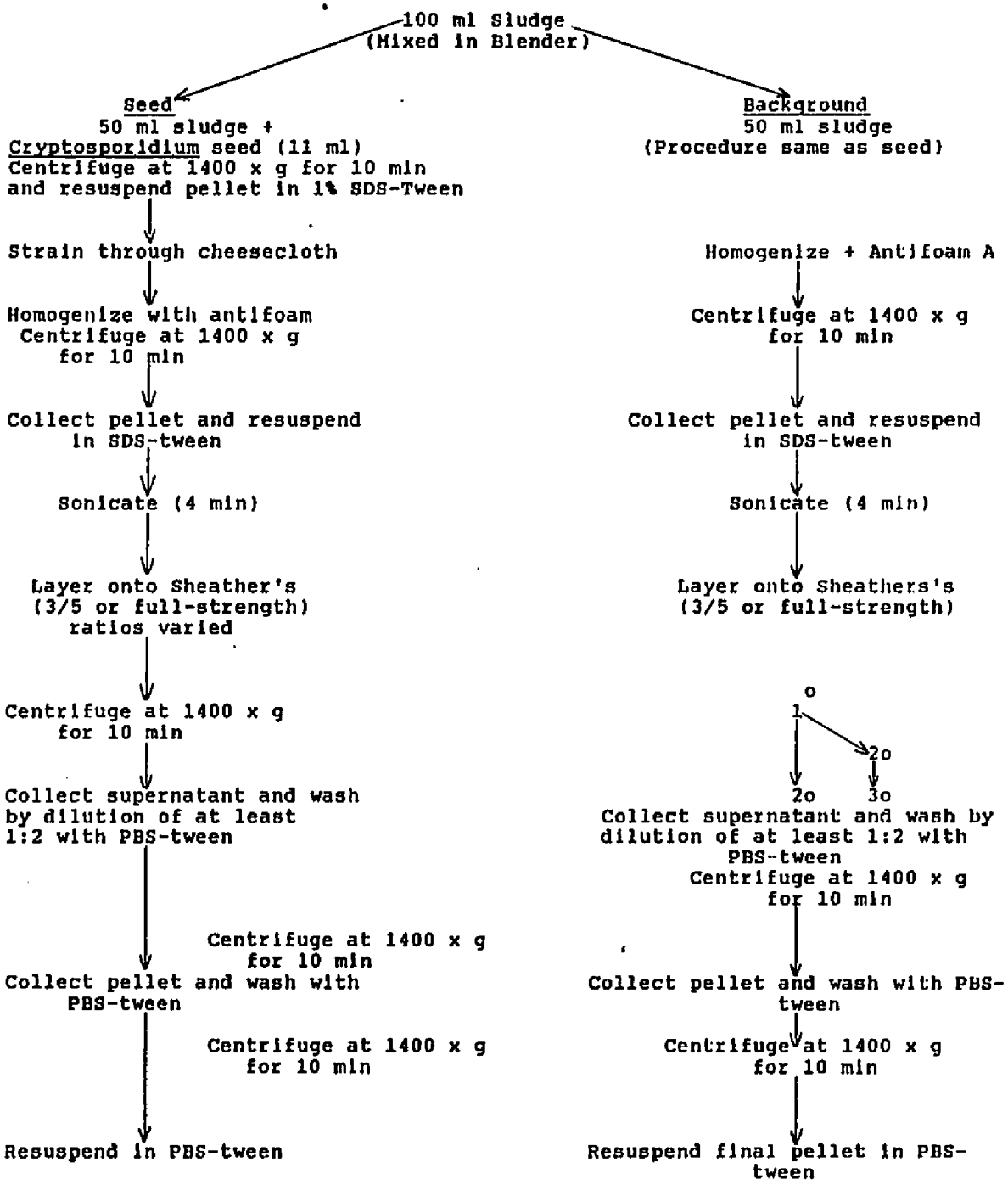

Figure 1. Method for Detection of Cryptosporidium in Sludge

two ml of oocysts and mixed to give a total volume of 22 ml. Eleven ml were removed after addition of 1% tween and placed in a 15 ml conical centrifuge tube to serve as the seed count (10^4 - 10^7 oocysts). The remaining 11 ml were seeded into 50 ml of sludge. The seed oocysts were centrifuged in a bench top centrifuge at 1400 x g for 10 minutes. The pellet (0.4 ml) was resuspended in 1% phosphate buffered saline (PBS) in tween to a volume of 4 ml.

Sludge samples used in this study were obtained from Boston, MA and were anaerobically digested. One hundred ml of sludge was mixed in a blender. Fifty ml was removed and placed in a 50 ml conical centrifuge tube and processed for the background count. The remaining 50 ml was seeded with 11 ml of tap water containing oocysts, mixed in a blender and placed into two 50 ml conical centrifuge tubes. All three tubes were centrifuged (1400 x g for 10 minutes) in a bench top centrifuge.

Pellet Processing

Supernatants were removed by vacuum aspiration. Pellets of the seeded sludge were combined and both the seeded and background pellets were resuspended in a detergent solution [deionized water with 1% Tween 80 and 1% sodium dodecyl sulfate (SDS)]. In some cases, samples were strained through cheesecloth. All samples were then homogenized for approximately 2 minutes at a low speed

(Vitris "45" Gardiner, NY). One drop of antifoam A (Sigma Chemical Co., St. Louis, MO) was added to remove all suds and facilitate recovery of the entire sample. The entire sample was then placed in a 50 ml conical tube. Samples were washed by centrifuging at 1400 x g for 10 minutes. Supernatants were aspirated off and pellets were resuspended in the detergent solution. The seeded and background sample tubes were kept separate and processed in the same manner. In all cases, samples were then placed in a water bath sonicator (Ultrasonic Cleaning System E-module, Branson Cleaning Equipment Co., Shelton, CT) for four minutes.

Sheather's flotation was used to concentrate oocysts. Following sonication a volume of sample (5 ml) was layered onto a volume of full strength Sheather's (500 gm sucrose, 320 ml distilled water and 9.7 ml liquid phenol), 3/5 Sheather's or both. A variety of volumes and sample to flotation ratios were used which included a 1:2, 1:3 and a 1:5. Flotation tubes were centrifuged at 1400 x g for 10 minutes, supernatants were recovered and the pellets were disposed of. Recovered supernatants were washed with 1% phosphate buffered saline (PBS) in tween by a dilution of at least 1:2 and centrifuged at 1400 x g for 10 minutes. Pellets were recovered and the excess aqueous solution was removed by vacuum aspiration. Pellets were once again washed in 1% PBS-tween by centrifugation at

1400 x g for 10 minutes. The excess aqueous solution was removed and pellets were resuspended up to a volume of 10 ml in 1% PBS-tween.

Oocyst Detection

One ml portions of the sludge sample were either filtered directly through a syringe fitted with 13 mm cellulose nitrate membrane filters with a pore size of 1.2 um or after diluting 1:10. Filters were stained with 0.1 ml of a working solution of the monoclonal antibody to the oocyst wall of Cryptosporidium. The filters were incubated at room temperature for 20 minutes. The filter was washed with PBS-tween to remove background fluorescence and mounted on a microscope slide in 1% glycerol-PBS. Oocysts were enumerated on the filter using epifluorescent microscopy.

Percent recoveries were determined by total number of oocysts recovered from the sludge minus the background number of oocysts divided by the total number of oocysts from the seed count.

Effects of Flotation Ratios on Percent Recoveries of Oocysts from Large Volumes of Sludges

The method for this set of experiments followed that outlined in the section on the general procedure for detection of Cryptosporidium in sludges. In order to determine whether larger volumes of sludge (50 ml)

decreased percent recoveries of oocysts, 100 ml volumes of sludge were seeded with 11 ml of a Cryptosporidium seed and mixed in the blender. The procedure included homogenization, sonication and the use of detergents. Fifty ml of sludge was layered onto 150 ml and 250 ml of 3/5 Sheather's solution to give a 1:3 and a 1:5 ratio, respectively. Sludges with different amounts of solids were used to determine if it affected percent recovery of oocysts. Percent recoveries were determined in the usual manner and background counts were taken into account.

Statistical Analysis

Prior to analysis, the data presented were transformed into either: a) log 10 y or; b) arcsin, depending on percent recoveries. Analysis of variance was used as described in Sokal and Rholf (1981).

Student t-tests were examined for some of the data to determine significant differences between means. Six variables: fecal streptococcus, total coliform, fecal coliform, percent solids, percent recoveries of oocysts and numbers of naturally occurring Cryptosporidium were analyzed for intercorrelation using the Cyber 175 and Dec-10 computers at the University of Arizona Computer Center with the Regran Program (Veldman, 1967), following $\log_{10} y$ transformations of most variables and arcsin transformations for percent recoveries. No transformations were done on the percent solids.

CHAPTER 3

RESULTS

Use of Membrane Filtration for Giardia Cyst Enumeration

The effects of membrane filter pore size on recoveries of cysts was tested and results are shown in Table 1. Counts were made on 3.0 um and 5.0 um pore size membrane filters. Filtrates were counted on a 1.2 um pore size filter to verify cyst retention. The filters were similar in recovery of Giardia, however, the 5 um pore size filter gave a cleaner sample which facilitated detection of the cyst. This size filter was chosen for microscopic examination of Giardia for the entire study.

Evaluation of Various Flotation Techniques for the Recovery of Giardia Cysts from Stool

Recoveries of Giardia cysts from stool using the various flotation media were evaluated and compared. Results are shown in Table 2 and percent recoveries averaged between 40.6% to 77% after five trials. Potassium citrate and percoll-sucrose gave the highest recoveries of 76.2% and 77%, respectively, however analysis of variance showed no significant difference in recoveries for each of the flotation media (Appendix 1).

Table 1. Retention of Giardia Cysts on Polycarbonate Membrane Filters

Filter Pore Size (um)	Replicate Counts	Filtrate Count[1]
3	31	0
5	26	
3	32	0
5	24	0
3	34	
5	14	

[1] Filtered through a 1.2 um pore size filter

Table 2. Recovery of Giardia (%) Using Various Flotation Media* with Detergents

Experiment Number	Potassium Citrate	Percoll-Sucrose	Full-strength Sheather's	4/5 Sheather's	Zinc Sulfate	3/5 Sheather's
1	47	28	38.5	31	21	22
2	84	93.5	37.6	57.8	77	61.5
3	69	142	109	101	145	62
4	64	62	54	53	30	30
5	117	59	110	97	59	27.5
Ave.	76.2	77	69.8	68	66.4	40.6

Centrifugation at 1400 x g for 10 minutes

* 1:10 - One ml sample layered on 9 ml flotation

Turbidities were also recorded for each flotation to evaluate the clarification of the sample (Table 3). Average turbidities were 106, 151, 119, 140, 160 and 137 NTU respectively for potassium citrate, percoll-sucrose, full-strength Sheather's, 4/5 Sheather's, zinc sulfate, and 3/5 Sheather's. Similarly, analysis of variance showed no significant difference in turbidities for each of the flotations (Appendix B).

Recovery of Giardia Cysts from Stool Using 4/5 Sheather's and Potassium Citrate without Detergents

The average percent recoveries of Giardia from 4/5 Sheather's was 35.6% and for potassium citrate was 41% (Table 4). Analysis of variance did not demonstrate a significant difference between the two flotations, however the use of detergents (Table 2) did increase recoveries significantly at the 95% confidence limits (Appendix C) when compared to recoveries in Table 4.

Effects of Sonication, Detergents and Low Speed Centrifugation (600 xg for 2 min) on Recovery of Giardia Cysts from Stool

Three trials were done with two different stool samples. Recoveries and turbidities were recorded (Tables 5 and 6). Trials 2 and 3 gave much lower turbidities due to a cleaner stool sample. Analysis of variance demonstrated no significant difference between the two

Table 3. Effects of Various Flotations on Sample Turbidity (NTU)

Trial Number	Potassium Citrate	Percoll-Sucrose	Full-strength Sheather's	4/5 Sheather's	Zinc Sulfate	3/5 Sheather's
1	130	145	180	230	300	200
2	90	265	125	190	180	190
3	110	83	85	71	125	75
4	112	120	110	120	120	120
5	88	140	93	90	73	100
Ave.	106	151	119	140	160	137

Table 4. Recovery of Giardia Cysts from Stool Using Sheather's and Potassium Citrate[1] Flotation Without Detergents

Trial	Percent Recoveries	
	4/5 Sheather's	Potassium Citrate
1	10.5	9.4
2	36.1	55.0
3	60.1	58.6
Averages	35.6	41.0

[1] 1:10 - One ml sample layered on 9 ml flotation.

Table 5. Recoveries of Giardia Cysts from Stool Using Sheather's Flotation[1,2] and Potassium Citrate Flotation[2] with Low Speed Centrifugation,[3] Sonication and Detergents

	Percent Recoveries	
Trial	4/5 Sheather's	Potassium Citrate
1	52.9	63.8
2	75.8	58.2
3	96.9	68.2
Averages	75.2	63.4

1 Full strength Sheather's 500 gm sucrose, 320 ml deionized water + 9.7 ml liquid phenol

2 1:10 ratio - One ml sample layered onto 9 ml flotation.

3 Low speed centrifugation = 600 x g for 2 minutes.

Table 6. Effects of Potassium Citrate and 4/5 Sheather's Flotation (1:10) on Sample Turbidities Using Detergents, Sonication and Low Speed Centrifugation

Trial	4/5 Sheather's	Potassium Citrate
1	118	111
2	20	30
3	21	10
Averages	53	50

flotations, however, a significant difference at the 95% confidence level was found between treatments (Appendix D) that is low speed centrifugation, sonication and detergents gave higher recoveries (Table 5) than, high speed centrifugation, no sonication and no detergents (Table 4).

Recovery of Giardia Cysts from Stool Using Various Flotation Ratios with Potassium Citrate and 4/5 Sheather's

Percent recovery averages of the 1:2, 1:3 and 1:5 ratios for potassium citrate and 4/5 Sheather's ranged from 46.6% to 56.6% and 78.9% to 83.1%, respectively (Tables 7 and 8). A 5 ml sample volume of a homogenized stool preparation was layered onto 10 ml, 15 ml and 25 ml of flotation to give a 1:2, 1:3 and a 1:5 ratio, respectively. Percent recoveries were compared in order to evaluate increased volumes and different flotation ratios. Analysis of variance showed no significant difference between ratios however, there was a significant difference at the 95% confidence level between flotations (Appendix E). The 4/5 Sheather's gave significantly higher percent recoveries for each of the ratios tested than did potassium citrate. Turbidities were also recorded and values are presented in Tables 7 and 8 for each of the flotation ratios. Turbidities for potassim

Table 7. Effect of Potassium Citrate Flotation Ratios on Turbidities and Recovery of Giardia

	Recovery of Giardia Cysts (%) Ratio of Potassium Citrate[1]			Turbidities (NTU) Ratio of Potassium Citrate[1]		
	1:2	1:3	1:5	1:2	1:3	1:5
1	57.3	29.7	46.2	23	15	30
2	29.1	39.7	36.4	18	16	16
3	83.3	70.5	83.3	50	46	48
Averages	56.6	46.6	55.3	30.3	25.7	31.3

[1] - 5 ml sample volumes were used

Table 8. Effect of Sheather's Flotation Ratios on Turbidity and Recovery of Giardia

Trial Number	Recovery of Giardia Cysts (%) Ratio of 4/5 Sheather's[1]			Turbidities (NTU) Ratio of 4/5 Sheather's[1]		
	1:2	1:3	1:5	1:2	1:3	1:5
1	79.8	87.2	90.2	55	45	47
2	93.8	90.5	78.5	46	42	40
3	75.2	59.0	80.6	35	43	41
Averages	82.9	78.9	83.1	45.3	43.3	42.7

[1] - 5 ml sample volumes were used

Table 9. Summary of the Recovery of Giardia from Stool Using Potassium Citrate and 4/5 Sheather's with Various Treatments

Flotation Type	Detergent	Sonication	Treatments 1400 X g for 10 min.	600 X g for 2 min	Ratios Ratio 1:10	Ratios 1:2, 1:3, 1:5	Average % Recovery
Potassium Citrate							
1	X		X		X		76.2
2			X		X		41.0
3	X	X		X	X		63.4 *
4	X	X		X		X	52.8
4/5 Sheather's							
1	X		X		X		68
2			X		X		35.6
3	X	X		X	X		75.2 *
4	X	X		X		X	81.6

* Average % recovery of combined ratios (1:2, 1:3 and 1:5)

X Denotes flotation treatments

citrate ranged from 25.7 to 31.3 NTU and 42.7 to 45.3 for 4/5 Sheather's.

Development of a Method for the Detection of Cryptosporidium Oocysts in Sludges

Various methods were used to recover Cryptosporidium from sludges and recoveries for all trials ranged from 3.8% to 54.2% with an average of 26.8% (Table 10). All methods incorporated the use of homogenization, sonication, detergents and Sheather's solution. Variations included the use of cheesecloth filtration, flotation ratios of 1:2, 1:3 or 1:5 and various volumes of sample processed (Table 11). Full-strength Sheather's was initially used which resulted in a sample that required further clarification (secondary and tertiary). The use of 3/5 Sheather's eliminated this problem. Ratios of 1:3 or 1:5 also improved the clarity of the sample. Cheesecloth filtration apparently decreased recoveries. When using 3/5 Sheather's, no cheesecloth filtration and a 1:3 flotation ratio, recoveries ranged from 16.4% to 54.2%.

The effects of sonication and cheesecloth filtration on the number of oocysts recovered from sludges was examined (Tables 12 and 13). Statistical analysis by the student t-test showed that sonication significantly ($p < .01$) increased numbers of oocysts recovered (Appendix F)

Table 10. Percent Recoveries of Cryptosporidium Oocysts from Anaerobically Digested Sludges

Sample	Total Oocysts Seed ($\times 10^3$)	Total Oocysts Recovered* ($\times 10^3$)	% Recovery
MA1	7440	2650	35.6
MA2	4640	759	16.4
MA3	152	76	50.0
MA4	968	233	24.1
MA5	11300	1920	16.9
MA6	3840	520	13.5
MA7	33.2	18	54.2
MA8	700	26.7	3.8
Average			26.8

* (Minus Background Counts)

Table 11. Percent Recovery of Cryptosporidium Oocysts from Sludges Using Various Sheather's Flotations and Volume Ratios

Sample Code Number	Type of Sheather's Flotation	Volume Sample Layered/ Flotation Volume ml	% Recovered
MA8	1° Full-Strength*	5/10	
	2° Full-Strength	50/100	
	3° 3/5	5/10	3.8
MA3	1° Full-Strength	5/25	
	2° 3/5	5/15	50.0
MA4	1° 3/5**	5/15	24.1
MA7	1° 3/5	5/15	54.2
MA6	1° 3/5**	5/25	7.7
	1° 3/5	5/25	13.5
MA2	1° 3/5	5/15	16.4
MA1	1° 3/5	5/15	35.6
MA5	1° 3/5	5/25	16.9

* Full-strength sheathers (500 grams sucrose, 320 ml distilled water and 9.7 ml liquid phenol)

** Cheesecloth filtration

Table 12. Effect of Sonication on the Number of Seeded Oocysts Recovered from Two Sludges

Sample Number	Sonication	
	With	Without
1	157	82
	106	56
	98	77
	110	59
2	175	97
	197	159
Averages	140.5	88.3

Table 13. Effect of Cheesecloth Filtration on the Number of Oocysts Recovered from Sludges

Sample Number	With ($\times 10^3$) filtration	Without ($\times 10^3$) filtration	Dry Weight g/100 ml
1	299	580	1.7
2	40	760	1.6
3	1050	2660	3.1
4	1960	1920	1.96
5	4	60	1.7
6	1	1	1.6
Average	559	997	

whereas cheesecloth filtration was not found to significantly decrease numbers of oocysts recovered (Appendix G).

The effects of using Sheather's flotation on the recovery of oocysts from large volumes of sludge was also examined. In a set of three trials, 50 ml of sludge sample was layered onto 150 ml and 250 ml of 3/5 Sheather's to give a 1:3 and a 1:5, respectively. All samples were sonicated but not filtered. Percent recoveries for the 1:3 ratio ranged from 2.7% to 15.5% with an average of 7.3% and for the 1:5 ratio ranged from 4.9% to 22.2% with an average of 12.9% (Table 14). Statistical analysis by the student t-test showed no significant difference between the two ratios examined (Appendix H). No correlation was found between recoveries and amount of solids in the sludge (Appendix I).

A total of eight samples of anaerobically digested sludge were examined for natural isolates of Cryptosporidium using the criteria of characteristic size, shape, and reaction of antibody to the oocyst. All eight samples were found to contain Cryptosporidium (Table 15). Concentrations of oocysts per gram dry weight ranged from 3.06×10^{2} to 3.87×10^{4} with an average of 7.52×10^{3} oocysts/gram dry weight. Estimated maximum

Table 14. Effect of Sheather's Flotation Ratios on Recovery of Oocysts from Large Volumes* of Sludge

	% Recovery of Cryptosporidium	
	Ratio of Sheather's Solution	
Experiment	1:5	1:3
1	11.6	3.8
2	4.9	2.7
3	22.2	15.5
Average	12.9	7.3

* 50 ml volume

Table 15. Indigenous Concentrations of Cryptosporidium in Sludges

Sample	Indigenous *Cryptosporidium* Oocyst Number/ gm. dry weight[1]	% Recovery of Trial	Estimated* Oocysts/ gm. dry weight
MA1	1,570	35.6	4,410
MA2	1,250	16.4	7,620
MA3	2,500	50.0	5,000
MA4	5,830	24.1	24,200
MA5	306	16.9	1,810
MA6	4,700	13.5	34,800
MA7	5,330	54.2	9,930
MA8	38,700	3.8	1,020,000

* Based on Percent Recoveries

1 Geometric mean = 3,098

Arithmetic Mean = 7,523

concentrations of oocysts per gram dry weight based on trial recoveries ranged from 1.81×10^{3} to 1.02×10^{6} (Table 15).

A summary of the microbial quality of the anaerobically digested sludges used in this study is presented in Table 16.

Table 16. Summary of the Microbial Quality of Anaerobically Digested Sludges

Sample	Total Coliform /100 ml	Fecal Coliform /100 ml	Fecal Strep-tococcus /100 ml	Cryptosporidium Oocysts/ gm dry weight	Dry Weight g/100 ml
MA8	9.3×10^{8}	1.5×10^{8}	4.3×10^{6}	3.87×10^{4}	3.1
MA3	2.8×10^{8}	9.3×10^{7}	3.9×10^{5}	2.5×10^{3}	8
MA4	2.4×10^{7}	9.2×10^{5}	1.5×10^{5}	5.83×10^{3}	2.4
MA7	2.3×10^{7}	2.3×10^{6}	2.3×10^{6}	5.33×10^{3}	0.75
MA5	4.3×10^{7}	2.1×10^{6}	4.3×10^{6}	3.06×10^{2}	1.96
MA6	2.3×10^{6}	3.6×10^{5}	4.2×10^{4}	4.7×10^{3}	1.7
MA2	4.29×10^{7}	2.4×10^{6}	2.4×10^{5}	1.25×10^{3}	1.6
MA1	1.49×10^{7}	9.4×10^{5}	2.3×10^{5}	1.57×10^{3}	12.7

CHAPTER 4

DISCUSSION

Evaluation of Flotation Media for the Recovery of Giardia Cysts

Few sludges have been examined for the presence of Giardia primarily because methods were not available. Thus various flotation media (six) were evaluated for the recovery of Giardia cysts from stool (Table 2) in an attempt to determine the most effective flotation procedure which might be useful for isolating Giardia from sludge samples.

Membrane filtration (filter pore size, 5 um) was utilized in this study since it was a reliable method for the detection and recovery of cysts. Techniques for the enumeration of cysts include the use of a hemocytometer for microscopic counting and the cover slip technique (American Water Works Association, 1985). The hemocytometer, however, may not be efficient when small volumes of sample are available for examination or when low concentrations of cysts are present. Membrane filtration may be a valuable tool for further studies with Giardia spp. and other similar organisms due to its excellent recovery rate, precision and good reproducibility (Spaulding et al., 1983). This method

enables larger volumes of sample to be examined within a defined area.

The flotation step is critical in recovering cysts of Giardia from samples. Flotation may affect recoveries of the organisms due to use of different density solutions. Recoveries may, therefore, be enhanced by determining the best flotation density which gives the greatest number of desired organisms suspended in the flotation gradient. Ideally, a flotation system which gives optimal recoveries and clarification is most desirable. Six flotation media were, therefore, evaluated for their efficiency of Giardia cyst recovery: potassium citrate, percoll-sucrose, zinc sulfate, full-strength Sheather's, 4/5 Sheather's and 3/5 Sheather's, (Table 2). Statistically, there was no significant difference between recoveries (Table 2) and turbidities (Table 3) after five trials (Appendix A and B), 4/5 Sheather's and potassium citrate were further evaluated. Since membrane filtration was used for the final sample clarification, removal of suspended material and turbidity are very important. Potassium citrate gave an average percent recovery of 76.2% with the lowest average turbidity of 106 NTU and was thus further evaluated. Sheather's solution was effective in recovering Cryptosporidium from sludges and was further evaluated for the recovery of Giardia on the pretext that one flotation could be used to isolate both organisms from

environmental samples and sludges. A dilution of the Sheather's solution was necessary since full strength Sheather's seems to cause a distortion of the Giardia cysts beyond recognition due to the high osmolarity of the solution (Fox et al., 1981). Zinc sulfate is commonly used to recover Giardia cysts. In this study, however, it did not purify the sample sufficiently or as well as potassium citrate (Table 3). This step is critical in cyst detection. The more sediment, the greater the possibility of decreasing numbers of cysts recovered due to association of cysts with larger particulates that may settle out.

Musial (1985) found that the use of detergents (SDS, tween 80) and sonication increased recoveries during flotation. The use of detergents (1% SDS-tween 80) during flotation was, therefore, examined. Statistical analysis showed a significant difference at the 95% confidence level when detergents were used (Appendix D). Average recoveries were significantly higher for potassium citrate (76.2%) and 4/5 Sheather's (68%) when detergents were used as compared to lower average recoveries of 41.0% and 35.6% for potassium citrate and 4/5 Sheather's, respectively, when no detergents were used (Table 9). In contrast to this, Satchwell (1986) found that the use of detergents (cetyl pyridinium chloride, SDS, and Nonidet P-40) was ineffective in increasing recoveries of Taenia from

sludges. Recoveries were apparently less in every case where detergents were added prior to sieving the samples. This may be due to the characteristics of the sludge (different solids content), which make it different from stool, or it may be due to the two different organisms recovered in each study and the different detergents used. Recoveries may also be less because detergents were not used at the flotation step or because antifoam was not used and thus microorganisms were trapped in foam. Detergents are thought to break up hydrophobic interactions, to eliminate clumping and to reduce adherence of cysts to surfaces resulting in increased recoveries. Sonication may increase recoveries by breaking up aggregates of cysts and sediment.

A combination of low speed centrifugation, sonication, and detergents significantly increased average recoveries for potassium citrate (63.4%) and 4/5 Sheather's (75.2%) when compared to average recoveries of 41.0% and 35.6% for potassium citrate and 4/5 Sheather's, respectively, when a combination of high speed centrifugation, no detergents and no sonication was used (Table 9). This is not surprising since the use of detergents and sonication would increase recovery of Giardia cysts. No significant difference was found between the two flotation solutions used (Appendix D).

Different flotation ratios were examined for potassium citrate and 4/5 Sheather's. A sample volume of 5 ml was layered on 10 ml, 15 ml and 25 ml of flotation media to give a 1:2, 1:3 and a 1:5 ratio, respectively. Analysis of variance showed no significant difference between ratios, however, 4/5 Sheather's ratios significantly increased recoveries when compared to potassium citrate ratios (Appendix E). Thus, 4/5 Sheather's may be more efficient as a means of processing environmental samples when larger volumes need to be examined due to lower concentrations of Giardia.

Optimal recoveries for both flotation media were obtained using detergents, low speed centrifugation, sonication and a 1:10 flotation ratio. Sheather's dilution (4/5) gave optimal recoveries under the same conditions, however, the flotation ratios were greater (1:2, 1:3, and 1:5), whereas potassium citrate recoveries were increased when high speed centrifugation, detergents and a 1:10 flotation ratio were used (Table 9). A 1:10 flotation ratio may increase recoveries but may not be useful in processing samples since only a small sample volume may be examined. Higher flotation ratios may be more efficient and beneficial, although recoveries are slightly lower. It is not known from the data presented whether different centrifugation speeds have a direct effect on recoveries of Giardia.

Different stool samples may give different recoveries depending on the type and clarity of the sample. The same may be true for different environmental samples. When choosing the best method, a balance must be met between recovery of the organism, clarity of the sample, and volume of the sample examined. If a sample has a high organic content, clarity of the sample may take precedence over optimal recoveries resulting in a cleaner sample with a lower efficiency of recovery.

This is merely a preliminary evaluation of flotation media for the recovery of Giardia cysts. Evaluations of more flotation procedures are clearly necessary. Increasing sample volumes, varying flotation ratios, and possibly seeding environmental samples with a high inoculum of Giardia cysts to determine efficiencies and compare them to recoveries of Giardia from stool. The algae and organics in the environmental samples may have an adverse effect on recoveries, greatly reducing them. Recoveries may vary greatly depending on the type of sample examined. These are areas that need to be investigated and researched in order to maximize our understanding of this organisms interactions in the environment. It is hoped that the method developed will be useful in ascertaining the most effective procedure for the isolation and detection of Giardia from the environment.

Development of a Method for the Detection of Cryptosporidium Oocysts in Sewage Sludge

Cryptosporidium is now considered as a cause of diarrhea in humans and can be transmitted by sewage contaminated water. In 1984, the first waterborne outbreak was reported whereby a sewage-contaminated drinking well was the culprit, however, no Cryptosporidium was isolated from the contaminated well (D'Antonio et al., 1985). Little is known about the organism's epidemiology, including its presence and prevalence in the environment, however large numbers of oocysts (10^3/gallon) have been detected in treated sewage effluents (Rose et al., 1986), thus it follows that the organism may also be present in sludges. This study was undertaken to develop a method for the detection of Cryptosporidium oocysts in sludges and to use the method to attempt to detect natural isolates. In an initial attempt to try and adapt the methods used for Cryptosporidium, to Giardia, flotation media for the recovery of Giardia from stools was also evaluated.

During development of a method to recover Cryptosporidium from water Musial (1985) found that the use of detergents (SDS, tween 80) and sonication increased recoveries during flotations. Tween 80 was added to break up hydrophobic interactions and perhaps to break up aggregates of oocysts to give more accurate counts of the number of oocysts present.

Various methods to compare recoveries of Cryptosporidium from sludges were examined (Table 11). Variables included cheesecloth filtration, flotation ratios (1:2, 1:3, 1:5) and flotation concentrations. The Sheather's flotation procedure was used in order to separate the oocysts from the extraneous material present in resulting pellets. Sheather's flotation is used routinely to separate and recover Cryptosporidium oocysts from organic debris and other microorganisms in feces and in this study for separation of oocysts from material and organic debris found in sludges.

The concentration using Sheather's flotation was thought to be a critical step in increasing or decreasing recoveries. Full strength Sheather's did not purify the sample or remove sediment sufficiently. This interfered in oocyst detection. An excess of organics which could not fall through the undiluted Sheather's due to their particular densities may have been responsible for this observation. Dilution of the full-strength Sheather's gave a specific gravity of 1.17 and was found to be adequate in purifying the sample and concentrating oocysts. Percent recoveries of oocysts from 3/5 Sheather's ranged from 7.7 to 54.2% (Table 11). Recoveries of 16.4%, 35.6% and 54.2% were obtained using 3/5 Sheather's, a 1:3 ratio and no cheesecloth filtration.

Sonication and cheesecloth filtration of the samples were also further examined. Sonication was found to significantly increase the number of oocysts recovered (Table 12) possibly by breaking up aggregates of oocysts and sediment particles. Statistically, cheesecloth filtration was not found to decrease numbers of oocysts recovered. Although percent recoveries did decrease slightly, a cleaner sample was obtained and the detection of oocysts was facilitated when utilizing cheesecloth filtration. The oocysts may possibly have associated with the larger particles in the sludge and were taken out during filtration resulting in slightly lower counts (Table 13). Cheesecloth filtration is recommended for use in environmental samples, depending on the solid content, since it may alleviate the problem of excessive organic debris. This filtration produces a sample which is cleaner, facilitates detection of oocysts, and does not seem to significantly alter recoveries. Cheesecloth filtration is necessary in processing composted sludge since it contains a high content of wood chips, debris and organics which may interfere in the detection of oocysts.

Although pH was not examined it may affect the possible interactions between oocysts and sediment particles by determining the charge on both. It is not known at what pH levels the oocysts are negatively or positively charged which would aid in determining the

types of interactions occurring. Further information on the characteristics of the oocyst wall and interactions with various sediments is needed to optimize recovery.

During most seeded studies, small volumes of sludge were processed. In some cases, it may be necessary to process large volumes in order to detect low concentrations of oocysts. The effects of large volumes of sludge on recovery of oocysts were examined. In this set of three trials a 50 ml volume of sludge seeded with 10^4 - 10^7 oocysts of Cryptosporidium was layered onto 150 ml and 250 ml of 3/5 Sheather's to give a 1:3 and a 1:5 ratio. Percent recoveries averaged low for the 1:5 (12.9%) and the 1:3 (7.3%) (Table 14). This may be due to the pelleting out of oocysts associated with the organics. The flotation gradient may not have been long enough to allow for oocysts to collect in the suspension. Thus with increased volumes perhaps ratios greater than 1:5 should be used.

No correlation was found between percent recovery of oocysts and solid content of the sludge (Appendix I). However, the greater solid content may possibly cause interference and more association with the oocysts thus making it more difficult to read samples on the filter.

Detection of Cryptosporidium Oocysts in Sludges

Attempts were made to determine the concentration of oocysts in digested sludges. Unseeded sludges were

processed in the same manner as the seeded sludge for each of the different methods examined. Cryptosporidium oocysts were isolated in all eight samples of anaerobically digested sludges. Identification was based on size, shape and reaction of the antibody to the oocyst wall as mentioned previously.

It is not known whether the estimates of Cryptosporidium concentrations per gram dry weight ranging from 3.06×10^{2} to 3.87×10^{4} (Table 15) are indeed typical levels found in anaerobically digested sludges. Regular sampling and monitoring of sludges should be done between various treatment processes to ascertain the effectiveness of the treatment and the prevalence of oocysts. For effective removal of any pathogens the sludge should be digested in a batch process whereby all the sludge has been at temperature X for time Y (Feachem et al., 1983) as opposed to a continuous digestion process.

The methods used in this study do not determine whether oocysts are viable. They may be inactivated in sludge but still maintain their physical integrity. The mere presence and detection of Cryptosporidium in sludges may render them a potential health hazard. No literature is available on the viability of Cryptosporidium. Studies of excystation to ascertain viability of Cryptosporidium

during sludge treatment may be required to ascertain whether these oocysts are indeed infectious.

The method developed is merely a beginning in the ability to detect the organism in sludge. It is hoped, however, that this will be a stepping stone in helping to determine the health significance of Cryptosporidium in sewage sludges.

Conclusions

1. Natural isolates of Cryptosporidium were recovered in sludge.
2. Of all the methods tested, 3/5 Sheather's, a 1:3 flotation ratio and no cheesecloth filtration gave these recoveries: 16.4%, 35.6% and 54.2% for Cryptosporidium.
3. Larger volumes (50 ml) of sludge gave decreased relative recoveries of oocysts.
4. No relationship was found between the presence of Cryptosporidium and coliform bacteria in sludge.
5. The use of detergents enhanced recoveries of Giardia from potassium citrate and 4/5 Sheather's.

6. Flotation ratios of 1:2, 1:3 and 1:5 significantly increased recoveries of Giardia from 4/5 Sheather's when compared to recoveries obtained from potassium citrate ratios.
7. A combination of sonication, low speed centrifugation and detergents significantly enhanced recoveries of Giardia from stool samples.
8. A balance must be met between clarity of the sample and relative recoveries depending on the type of sample.

APPENDIX A

SUMMARY OF ANOVA: RECOVERY OF *GIARDIA* (%) USING VARIOUS FLOTATION MEDIA WITH DETERGENTS

Summary of Anova: Recovery of Giardia (%) Using Various Flotation Media with Detergents

Source	Degrees of Freedom	Sum of Squares	Mean Squares
Total	29	1.6420	-----
Trials	4	0.9352	0.2338
Solutions	5	0.2733	0.0547
Error	20	0.4335	0.0217

F Ratios

for trials/error = 10.77
for solutions/error = 2.52

Critical F Values

for trials/error F 4/20 = 2.87
for solutions/error F 5/20 = 2.71

The F value for the trials exceeds the critical F value, therefore, there is a significant difference between trials, however, this is not relevant.

APPENDIX B

SUMMARY OF ANOVA: EFFECTS OF VARIOUS FLOTATIONS ON SAMPLE TURBIDITY

Summary of Anova: Effects of Various Flotations on Sample Turbidity

Source	Degrees of Freedom	Sum of Squares	Mean Squares
Total	29	96492.667	-----
Trials	4	54061.0000	13515.2500
Solutions	5	9944.2667	1988.8533
Error	20	32487.4000	1624.3700

F Ratios

for trials/error = 8.32
for solutions/error = 1.22

Critical F Values

for trials/error F 4/20 = 2.87
for solutions/error F 5/20 = 2.71

The F value for the trials exceeds the critical F value, therefore, there is a significant difference between trials.

APPENDIX C

SUMMARY OF ANOVA: RECOVERY OF *GIARDIA* CYSTS FROM STOOL USING POTASSIUM CITRATE AND 4/5 SHEATHER'S WITH AND WITHOUT DETERGENTS

Summary of Anova: Recovery of Giardia Cysts from Stool Using Potassium Citrate and 4/5 Sheather's with and without Detergents

Source	Degrees of Freedom	Sum of Squares	Mean Squares
Total	15	1.463790	-----
Samples	3	.484085	
Solutions	1	.011556	.011556
Treatment	1	.471175	.471175
Solution X Treatment	1	.001354	.001354
Error	12	.979705	.081642

F Ratios

for solutions/error = .14154
for treatment/error = 5.77
for solution X treatment/error = .01658

Critical F Values

for solutions/error F 1/12 = 4.75
for treatment/error F 1/12 = 4.75
for solutions X treatment/error F 1/12 = 4.75

The F value for the treatment exceeds the critical F value, therefore, there is a significant difference between treatments (with and without detergents were the two treatments).

APPENDIX D

SUMMARY OF ANOVA: RECOVERY OF *GIARDIA* CYSTS FROM STOOL USING SHEATHER'S AND POTASSIUM CITRATE FLOTATION WITH VARIOUS TREATMENTS

Summary of Anova: Recovery of Giardia Cysts from Stool Using Sheather's and Potassium Citrate Flotation with Various Treatments

Source	Degrees of Freedom	Sum of Square	Mean Square
Total	11	3144.7214	-----
Solution	1	32.1391	32.1391
Treatment	1	1263.1647	1263.1647
Solution X Treatment	1	118.2047	118.2047
Error	8	1731.2129	216.4016

F Ratios

for solution X treatment/error = 0.5462
for treatment/error = 5.8371
for solution/error = .1485

Critical F Values

F 1/8 = 5.32

The F value for the treatment exceeds the critical F value, therefore, there is a significant difference between the two types of treatment. (Low speed centrifugation, sonication and detergents vs. high speed centrifugation, no sonication and no detergents were the two treatments.)

APPENDIX E

SUMMARY OF ANOVA: EFFECTS OF POTASSIUM CITRATE AND SHEATHER'S FLOTATION RATIOS ON RECOVERIES OF *GIARDIA*

Summary of Anova: Effects of Potassium Citrate and Sheather's Flotation Ratios on Recoveries of Giardia

Source	Degrees of Freedom	Sum of Square	Mean Square
Total	17	3654.7581	-----
Solutions	1	1359.6878	1359.6878
Ratios	2	99.0903	49.5451
Solutions X Ratio	2	30.3221	15.1610
Error	12	2165.6579	180.4715

F Ratios

for solutions X ratio/error = .0840
for ratios/error = .2745
for solutions/error = 7.5341

Critical F Values

for solutions X ratio/error F 2/12 = 3.89
for ratios/error F 2/12 = 3.89
for solutions/error F 1/12 = 4.75

The F ratios for the solutions exceeds the critical F value, therefore, there is a significant difference between the two solutions (potassium citrate and 4/5 Sheather's were the two solutions).

APPENDIX F

STUDENT T-TEST: EFFECTS OF SONICATION ON THE RECOVERIES OF OOCYSTS FROM SLUDGE

Student t-test: Effects of Sonication on the Recoveries of Oocysts from Sludge

Sonication		
With	Without	Ratio
157	82	1.91
106	56	1.89
98	77	1.27
110	59	1.86
175	97	1.80
197	159	1.24

Ra = 1.66

S^2_y = variance = .1007

SD = .3173

SE = .1295

t = 5.0965

T x .05 = 2.571

: p < .05 and reject null hypothesis.

Null Hypothesis: If there is no significant difference between the two columns, the ratios should be near or close to 1. Reject hypothesis since this is not the case.

APPENDIX G

STUDENT T-TEST: EFFECTS OF CHEESECLOTH FILTRATION ON THE RECOVERY OF OOCYSTS FROM SLUDGES

Student t-test: Effects of Cheesecloth Filtration on the Recovery of Oocysts from Sludges

Cheesecloth Filtration		
With ($\times 10^3$)	Without ($\times 10^3$)	Ratio ($\times 10^3$)
299	580	1.94
40	760	19
1050	2660	2.53
1960	1920	.98
4	60	15
1	1	1

Ra = 6.74

Variance = $S^2 y$ = 65.09

Standard Deviation = SD =18.07

SE = 3.29

t = 1.74

t .05 = 2.571

Thus there is no significant difference between the two treatments and accept null hypothesis.

Null Hypothesis: If there is no significant difference between the two columns the ratios should be near or close to 1.

APPENDIX H

STUDENT T-TEST: EFFECTS OF SHEATHER'S FLOTATION RATIOS ON RECOVERY OF *CRYPTOSPORIDIUM* OOCYSTS FROM LARGE VOLUMES OF SLUDGE

Student t-test: Effects of Sheather's Flotation Ratios on Recovery of Cryptosporidium Oocysts from Large Volumes of Sludge

Ratio of Sheather's Flotation		
1:5	1:3	Ratio
11.6	3.8	3.05
4.9	2.7	1.81
22.2	15.5	1.43

Variance = S^2_y = 0.7178

Standard Deviation = SD = .8472

Standard Error = SE = .4891

t = 2.24

t .05 = 4.303

Thus, there is no significant difference between the ratios examined and the null hypothesis is accepted.

Null Hypothesis: If there is no difference between the two columns, the ratios should be near or close to 1.

APPENDIX I

CORRELATION COEFFICIENTS FOR 6 VARIABLES MEASURED IN 8 DIFFERENT SLUDGE SAMPLES

Correlation Coefficients for 6 Variables
Measured in 8 Different Sludge Samples

	Solids	Arcsin % Recoveries	Total Coliform	Fecal Coliform	Fecal Strepto-coccus	Cryptosporidium (Natural Isolates)
Solids	1.0000	.3502	-.0979	.1390	-.1923	-.1493
Arcsin % Recoveries		1.0000	-.1414	-.0632	-.0592	-.2525
Total Coliform			1.0000	.9510	.6406	.3445
Fecal Coliform				1.0000	.5333	.4230
Fecal Streptococcus					1.0000	.0466
Cryptosporidium (Natural Isolates)						1.0000

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